DEVELOPMENT OF POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) BASED TECHNIQUES FOR MEAT IDENTIFICATION

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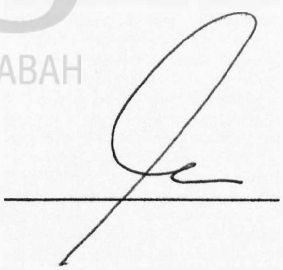

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ABSTRACT

DEVELOPMENT OF POLYMERASE CHAIN REACTION-RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) BASED TECHNIQUES FOR MEAT IDENTIFICATION

The primary objective of the research was to develop technique to identify various types of meats and to determine the detection of threshold levels of the technique. This technique is based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) of a conserved region in the mitochondrial (mt) cytochrome *b* (cyt *b*) gene for meat identification and authentication. Firstly, meat samples of pork, lamb, ostrich, cattle, buffalo, chicken and turkey were sampled randomly from commercial establishment in Sabah. The genomic DNA of known identities of meats were extracted and were subjected to PCR amplification by using the specific primers CYTb1 and CYTb2 targeting the mt cyt *b* gene. PCR amplification generated an amplicon with approximate size of 360 bp. The amplicon was cloned onto a TOPO® TA 2.1 plasmid, and sequenced. Sequences derived from these meats were aligned using SDSC biology workbench to determine homologous regions. The results of the alignment confirmed the identities of the meat species, and these sequences were used as references for the subsequent meat analyses and for future meat identification. Secondly, meat species identification was conducted by digesting the amplicons with a range of restriction endonucleases namely, *Alu*I, *Bsa*JI, *Bst*NI, *Bst*UI, *Nsi*I, *Rsa*I, *Taq*I which generates species-specific eletrophoresis banding patterns. The PCR-RFLP profile of all the species tested were used to construct a reference library and to provide a significant data for inter- and intra-species identification for this study. Thirdly, the threshold of detection using the PCR-RFLP method was tested using blended pork and chicken, or pork and beef which were mixed in different percentages, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1%, 5%, 10%, 20%, and 100% respectively. Results indicated that the PCR-RFLP method was sensitive enough to detect down to 0.1% of meat contaminant. Finally, processed meat products procured from various supermarkets were analyzed and authenticated using the technique developed and PCR conditions optimized in this study. The results of the analyses indicated that some of the meat labels did not reflect the content as claimed by the manufacturer. For instance, one of the processed meat products tested which was labeled as beef product was found to be contaminated by chicken meat while the raw beef were clearly not as it is claimed.

ABSTRAK

Objektif utama kajian ini adalah menghasilkan suatu kaedah molekular yang spesifik untuk mengenalpasti jenis-jenis daging dan menentukan tahap kesensitifan kaedah ini untuk mengesan kehadiran daging. Teknik ini adalah berdasarkan analisis 'polymerase chain reaction-restriction fragment length polymorphism' (PCR-RFLP) kawasan terpelihara dalam gen mitokondria (mt) sitokrom b (cyt b) untuk pengenalpastian jenis daging dan ketulenannya. Pertama sekali, sampel daging didapati secara rawak dari pasar dan pasaraya komersil di Sabah. Genomik DNA daging yang diketahui diekstrak dan diamplifikasikan dengan analisis tindakbalas rantaian polimerase (PCR) menggunakan primer spesifik CYTb1 dan CYTb2 yang menyasar gen mt cyt b. Amplifikasi PCR ini menghasilkan amplikon dengan saiz kira-kira 360 pasangan bes. Hasil PCR diklonkan ke dalam plasmid TOPO® TA 2.1 dan dijujuk. Jujukan DNA yang didapati daripada daging-daging ini disusun menggunakan program 'SDSC biology workbench' untuk menentukan kawasan homologi. Keputusan yang didapati mensahkan spesis daging tersebut dan jujukan DNA yang diperolehi dijadikan sebagai rujukan untuk analisis daging yang seterusnya dan juga untuk identifikasi daging di masa hadapan. Identifikasi spesis ditentukan dengan memotong amplikon-amplikon yang diperolehi dari hasil PCR dengan tujuh jenis enzim pembatasan (AluI, BsaJI, BstNI, BstUI, NsiI, RsaI dan TaqI) yang menghasilkan jalur DNA yang spesis-spesifik. Semua profil PCR-RFLP daging-daging yang dikaji dijadikan sebagai rujukan dan untuk menghasilkan data yang signifikan bagi identifikasi inter- dan intra- spesis dalam kajian ini. Ketiga, tahap kesensitifan kaedah PCR-RFLP ini ditentukan dengan menganalisa campuran daging babi dan ayam atau daging babi dan lembu dalam peratusan yang berbeza (0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 1%, 5%, 10%, 20%, dan 100%). Keputusan yang diperolehi membuktikan bahawa tahap kesensitifan kaedah ini untuk mengesan kehadiran daging asing dapat mencecah serendah 0.1%. Produk daging terproses juga dianalisa dengan menggunakan kaedah yang sama untuk mengenalpasti ketulenannya. Keputusan yang diperolehi menunjukkan bahawa terdapat daging terproses yang tidak menyatakan maklumat yang betul seperti yang tertera di atas label. Contohnya, salah satu daripada produk daging terproses yang dikaji yang berlabelkan daging lembu didapati mengandungi daging ayam di dalamnya.

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ABBREVIATION

α	Alpha
β	Beta
λ	Lambda
%	Percent
°C	degree Celsius
μg	microgram
μl	microlitre
μM	micromolar
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine Serum Albumin
bp	base pair
cyt *b*	cytochrome *b*
CO_2	Carbon dioxide
ddH_2O	double distilled water
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium bromide
g	gram
h	hour
kb	kilobase pair
KCl	Potassium chloride
kDa	Kilodalton
LB	Luria Bertani
M	molar
mg	milligram
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate

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min	minutes
ml	mililitre
mM	millimolar
mtDNA	mitochondrial DNA
NaCl	Sodium chloride
NADH	nicotinamide adenine dinucleotide, reduced form
O_2	Oxygen
pmole	picomole
RAPD	Random Amplified Polymorphic DNA
RE	Restriction Enzyme
RNase	Ribonuclease
rpm	revolution per minute
rRNA	ribosomal Ribonucleic acid
s	second
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TE	Tris-HCl EDTA
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
tRNA	transfer ribonucleic acid
U	unit
UV	ultra violet
V	volt

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CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The current demand for halal food in the global market has raised issues about the authenticity of some of the meat and meat products in the market, particularly in relation to uncertain and improper labeling, adulteration and substitution of cheaper meat to produce meat products, or the strict dietary limitation of various religions such as 'kosher' food for Jews and Muslims prohibition of pork consumption. There are also some who practice on vegetarian diet as well as those who are allergic to certain food component and are concerned about their healthcare.

The context of halal in this study is merely referred to the non-inclusion of porcine materials and not the method of slaughtering the animal. In the production of halal meat, raw and processed meats are separated from non-halal meat such as pork. Nevertheless, contaminations from other meats sometimes occur unintentionally or accidentally. Mislabeling is also an issue where pork or other meats are present in meat products labeled as halal or perhaps cow meat is substituted with cheaper buffalo meat.

In view of the fact that the demand for halal food is increasing rapidly in the global market, the government is currently active in exploring its potential in becoming one of the Global Halal Food Hub by setting up more than 10 integrated Halal hubs in the country under the 9th Malaysia plan. Its aim is to act as a regional hub to export the halal food to other Islamic countries. In July 2002, Malaysia has been chosen by Australia to manufacture halal food products for their market (www.foodproductiondaily.com, 2002). In September the same year, the government of Malaysia proposed the setting up of a halal food hub for the processing and packaging of Muslim food in corporation with companies from China

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and the Middle East (www.foodproductiondaily.com, 2002). In June 2003, New Zealand Minister for Trade Negotiations and Agriculture, Jim Sutton had stated that New Zealand, one of the major producers of meat products, is keen to pursue the concept of Malaysia as a hub for halal meat products (The Star, 2003). With all these international investments on halal food products which will increase Malaysia's economic growth and decrease the reliability on imported meat products, it is more important that Malaysia have a scientific means to ensure the authenticity of meats it produces. This would allow manufacturer and enforcement agencies such as the laboratories attached to the Department of Islamic Development Malaysia (JAKIM) to carry out analyses to detect contamination and authenticate a meat product in order to meet the requirement of international standards. Thus, it is necessary to develop good analytical procedures and methods for meat authentication to fight adulteration of meats in meat products. Additionally, meat detection technique that detects meat contaminant in food is also advantageous to followers of other religions and clinical patients with strict diets.

Several meat identification techniques based on protein from meats have been developed. But, these techniques have many disadvantages because they are not very sensitive and are unable to detect severely heat-treated meat due to protein denaturation at processing. Upon heating, proteins will be apt to become insoluble, which means they are complicated to extract and evaluate on gels. Moreover, immunological methods are based on shape identification, and the native shape of proteins is lost when they are heated to cooking temperatures during processing (Wong, 2005). Hence, researchers are working to develop a more versatile and simpler yet accurate and rapid method to determine the species of meat in meat products, especially in cooked-meat products (Brodmann & Moor, 2003; Saez *et al.*, 2004; Wintero *et al.*, 1990; Zhang *et al.*, 1999).

Alternatively, meat can be authenticated by using molecular techniques based on DNA. These techniques are sensitive and accurate because DNA is more resistant to heat (Unseld *et al.*, 1995), and the information provided by DNA is also much greater than protein (Wolf *et al.*, 2000).

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One of the genes in the mitochondrial DNA that has been shown to be useful in species identification is the cytochrome *b* gene (cyt *b*) (Comi *et al.*, 2005; Farias *et al.*, 2001; Johnson & Sorenson, 1998). It is found in the mitochondrial genome of all meats that have polymorphism among different animals. The cyt *b* gene can be easily isolated after PCR amplification obtained from all types of meats using two universal PCR primers. The cyt *b* genes from different types of meats have polymorphic DNA sequence. Therefore by digesting the amplified cyt *b* DNA fragment with restriction enzymes, they are expected to produce DNA fragments of different sizes that will show different DNA banding pattern on the agarose gel. The different banding patterns of DNA on gel and its uniqueness to certain meat will differentiate various meats including pork meat (Aida *et al.*, 2005).

The aim of this project is to develop a molecular technique based on Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) to identify the identity of several animal species. PCR is proved to be extremely sensitive for detection of minute amounts of DNA, specifically amplifying a target region of template DNA (Saiki *et al.*, 1988). In cases where there are conflicting or ambiguous RFLP patterns, sequencing of the cyt *b* gene will be conducted to ensure a more precise identification of a meat species.

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1.2 OBJECTIVES

The objectives of this project are:

(a) To develop a PCR-RFLP of the cytochrome *b* gene to identify the identity of various meats.

(b) To sequence the cytochrome *b* gene of various meats, and to be used as reference standards.

(c) To determine the sensitivity of these techniques for detection of the presence of pork in meat mixtures.

(d) To authenticate meat-based processed food product for any contamination from foreign meat.

CHAPTER 2

LITERATURE REVIEW

2.1 PROTEIN-BASED ANALYSIS FOR FOOD AUTHENTICATION

There are several methods for meat speciation which includes the use of lipids, proteins or DNA (Bellagamba *et al.*, 2001; Brodmann, 2002; Dooley *et al.*, 2004). The conventional methodology used for the determination of species of origin in meat products has been predominantly based on the immunochemical (Dooley *et al.*, 2004; Lenstra *et al.*, 2001; Carrera *et al.*, 1996) and electrophoretic analysis (Rehbein, 1990; Babiker *et al.*, 1981; Kim & Shelef, 1986) of proteins.

Isoelectric focusing (IEF) gel electrophoresis can be used to separate proteins on polyacrylamide gel which has a pH-gradient. The separated proteins are subsequently stained with coomassie blue or silver staining reagent (Brodmann, 2002; Jaussen *et al.*, 1990). IEF method on caviar proteins was used to differentiate black caviar lots in the 1980s (Keyvanfar *et al.*, 1988; Chen *et al.*, 1996; Rehbein, 1985). Although IEF has successfully discriminated raw meat and fish, it was not suitable for processed or heat-treated meat products due to the rapid degradation of most soluble proteins under such conditions (Rehbein *et al.*, 1990; Jemmi & Schlosser, 1993). Soluble muscle proteins degrade easily and quickly under high pressure and temperature thus prevents identification of species, as these techniques require large amount of high quality protein (Cheng *et al.*, 2003).

Moreover, the analysis by immunoassay, based on the use of antibodies rose against a specific protein, often present cross-reaction with closely related species (Meyer *et al.*, 1995; Berger *et al.*, 1988). These approaches not only needs specially trained expert but also are time-consuming. Immunological methods including enzyme linked immuno-sorbent assay (ELISA) (Chen *et al.*, 1998; Hsieh *et al.*, 1996) which sensitively identified the species of origin of raw meats were significantly less

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sensitive in severely heat-treated materials because of the alteration of specific epitopes (Hoffman, 1996; Gouli *et al.*, 1999; Calvo *et al.*, 2001). Although immunoassay kits provided for qualitative detection of species are available commercially, but differentiation between poultry species is not applicable (Dooley *et al.*, 2004). It has also been an issue that contamination of meat with blood from other species could lead to false results.

2.2 DNA-BASED ANALYSIS FOR FOOD AUTHENTICATION

DNA based analyses are widely used in many medical fields, and are becoming more popular for the differentiation and identification of food and food products. Sequences of mitochondrial or genomic DNA has been analyzed for various animals such as domesticated animals (Burgener & Hübner, 1998; Matsunaga *et al.*, 1999; Aida *et al.*, 2005; Bellagamba *et al.*, 2001; Bravi *et al.*, 2004; Dooley *et al.*, 2004; López-Andreo *et al.*, 2005), fish (Cheng *et al.*, 2001; Hsieh *et al.*, 2005; Quinteiro *et al.*, 1998; Carrera *et al.*, 1999), game species (Wolf *et al.*, 1999), and caviar (Birstein *et al.*, 1998) by using either universal or specific primers for the study of inter- and intraspecies relationships.

There are several advantages of DNA analysis methods because DNA is a relatively stable molecule thus allowing for the analysis of processed and heat-treated food products (Beneke & Hagen, 1998; Unseld *et al.*, 1995). The genetic information contained in DNA is greater than protein due to the degeneracy of the genetic code as one goes from DNA to protein (Wolf *et al.*, 2000). Other than that, DNA can be easily extracted from all kinds of tissue due to the ubiquity of DNA in every type of cell (Wolf *et al.*, 2000). Therefore, DNA can be isolated from various animal tissues such as muscle, blood, bones or fat tissues (Meyer *et al.*, 1995; Parson *et al.*, 2000, Aida *et al.*, 2005).

Hence, more and more DNA-based approaches such as single strand conformation polymorphism (SSCP) (Rehbein *et al.*, 1997; Comi *et al.*, 2005) and denaturing gradient gel electrophoresis (DGGE) (Comi *et al.*, 2005) have replaced protein-based approaches.

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2.3 MEAT COMPONENT AND ITS PROCESSING

Muscle cells are multinuclel cells that contain mitochondria, myoglobin, myofibrils (actin and myosin), and glycogen in the sarcoplasm. Active muscles are known to have higher number of mitochondria than less active cells because their energy requirement is higher.

Muscle tissues are very high in protein, containing all of the essential amino acids, which makes it an important source of food in the human diet. The nutrients in meat and meat products are digestible and readily available. It is estimated that 16% of the total calories consumed in the world came from animal products while the remaining 84% are of plants (Damron, 2003). Apart from being a good source of protein, meat and meat products also contain high amount of micronutrients such as selenium, folate and zinc which were reported to be cancer-preventive component by Biesalski (2002). Additionally, meat also acts as an essential provider of dietary ions and is rich with vitamin A and vitamin B12 necessary for healthy growth and development in children, as well as for maintaining a good health in adults. However, the consumption of meat as an important component of a balanced diet varies according to age and performance group, ranging from children to pregnant women, and from physically active individuals to senior citizens as well as for those with health problems. The fat content in meat varies accordingly depending on the species and breed of animal, their anatomical body parts, and the methods of butchering and cooking.

In most developing countries, slaughtering, processing and distribution of meat for human consumption are handled by meat packing industries. Meats are processed into various forms to produce steaks, stews, dried or simply fresh raw meat. It can be ground or minced and formed into patties like burgers, loaves, or sausages. In an attempt to store meats for longer period of time and to avoid spoilage, the meats are preserved and cured by smoking, pickling, preserving in salt or brine. Meats are also marinated, barbecued, boiled, roasted and fried. Some of the processed meats such as sausages and burgers are often spiced and seasoned to provide additional flavor in them, while some are molded or pressed and canned.

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2.4 THE STRUCTURE OF THE MITOCHONDRIA

Mitochondrion (Greek: *mitos*, thread + *chondros*, granule) is an organelle that is found in most eukaryotic cells, including plants, animals, and fungi. Mitochondria (plural for mitochondrion) were first seen as granules in muscle cells in 1850 by Kollicker (Logan, 2003).

Mitochondria vary in size and shape, depending on their source and metabolic activity (Voet *et al.*, 1999). They are generally rod-shaped organelles with dimensions of around 0.5 x 1.0 μm, about the size of a bacterium. Mitochondria have a double membrane structure with the inner membrane being highly folded. This results in a sac with two inner compartments which are separated by the inner membrane. The first compartment is between the outer and inner membranes, the second compartment is inside the inner membrane with a much larger internal matrix (Figure 2.1). The matrix is a gel-like solution that contains highly specialized proteins as well as substrates, nucleotide cofactors, and inorganic ions. The matrix also contains the mitochondrial genetic machinery (DNA, RNA and ribosomes) that generates several mitochondrial proteins (Voet *et al.*, 1999). The outer mitochondrial membrane contains many channels formed by the protein porin that allows free diffusion of molecules of up to 10 kDa. The inner membrane with the cristea contains enzymes which perform reactions required for the final step in aerobic respiration. It is permeably only to O_2, CO_2, and H_2O. In addition, the inner membrane utilizes numerous transport proteins that control the passage of metabolites such as ATP, ADP and phosphate.

Mitochondria are cellular organelles responsible for energy production in eukaryotic cells. They perform oxidative phosphorylation which involves the oxidation of carbohydrate intermediates, fat and amino acids that releases adenosine triphosphate (ATP) in the presence of oxygen. This is possible by forming a pH and electrical gradient known as the chemiosmotic gradient across the inner mitochondrial membrane (Juneja, 2002; Robinson *et al.*, 2003; Karp, 1999; Fairbanks & Andersen, 1999). Without mitochondria, higher animals would possibly not exist because their cells would only be able to obtain energy from anaerobic respiration which is a process in the absence of oxygen.